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Allozyme and isozyme variation in seven southern African Elephant-shrew species

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Abstract

Analysed were the allozyme and isozyme variation in seven species of southern African elephant-shrews. Allozyme and isozyme patterns obtained were species-specific. The data obtained showed a vast degree of divergence amongst the members of the genus *Elephantulus*, with Nei's genetic distance values ranging from 0.571 to 0.810 while the degree of divergence between *Macroscelides proboscideus* and *Petrodromus tetradactylus* was rather low, with a Nei's genetic distance value of 0.323. Genetic heterozygosity ranged from 0.000 to 0.055 while polymorphism ranged from 0.00% to 12.50%. Based on the results of UPGMA analysis *E. brachyrhynchus* was retained in the genus *Elephantulus*.

Introduction

Elephant-shrews are an order of small insectivorous mammals, endemic to Africa. Despite this restricted distribution, their classification both at the ordinal and species level has been the centre of much debate (Corbet 1978; Butler 1978, 1995). Earlier investigators have classified elephant-shrews as Tupaiidae, Hyracoidea and Lagomorpha (McKenna 1975; Tolliver et al. 1989). They have since been placed in an order of their own, Macroscelidea (Meester et al. 1986; Skinner and Smithers 1990).

The family, Macroscelididae, is divided into two subfamilies namely Rhynchocyoninae, which consists of one genus, *Rhynchocyon*, with three species and Macroscelidinae, comprising three genera namely, *Petrodromus*, *Macroscelides* and *Elephantulus* (Corbet and Hanks 1968). Both *Macroscelides* and *Petrodromus* include a single species whereas the genus *Elephantulus* comprises 15 species (Corbet 1974). Members of the Macroscelidinae, particularly those belonging to the genus *Elephantulus*, are widely distributed in Africa especially in the southern African subregion.

Subtle morphological differences in conjunction with biogeographical information has been used to delimit the different species of this genus. One *Elephantulus* species, *E. brachyrhynchus*, differs from the other members in this genus by having a third molar tooth on the lower jaw. Based on the possession of this feature, *E. brachyrhynchus* was initially placed in a genus of its own, *Nasilio*. The retention of the third molar tooth has since been identified as a primitive feature and therefore should not be used as the only feature to delimit genera (Corbet and Hanks 1968; Butler 1984). Using this argument *Nasilio* has been assigned to the genus *Elephantulus* (Corbet and Hanks 1968; Corbet 1974, 1995).

Most research involving these animals has been directed towards solving the ordinal question of these animals with very little research being conducted to either solve the tax-

onomy at the species level or to understand the biology of these animals. The research presented in this study forms part of a larger investigation aimed at understanding the biology and the evolutionary history of elephant-shrews. This portion of the study had three basic aims. The first aim was to assess the intra- and interspecific allozyme/isozyme variations of seven elephant-shrew species with the hope of identifying species-specific markers, second, it was hoped to establish on the basis of allozyme/isozyme analysis whether *E. brachyrhynchus* belongs to the genus *Elephantulus* or in a genus of its own. The final aim was to compare the phylogeny based on the allozyme/isozyme data with that based on morphological characters in order to assess which gave a clearer picture of the systematics and evolutionary history of these animals.

Material and methods

Animals

Seven southern African elephant-shrew species were investigated. The *Elephantulus* species (*brachyrhynchus*, *edwardii*, *intufi*, *myurus*, and *rupestris*) and *M. proboscideus* were trapped in Elliot small mammal traps while *P. tetradactylus* were caught in box traps previously used for trapping small carnivores. The localities at which the animals were trapped are shown in table 1. Once brought in from the field, the animals were treated for ectoparasites and housed in the departmental animal house in glass cages.

Table 1. Localities at which the elephant-shrew species were trapped.

Species	Area	Grid reference
E. brachyrhynchus	Princess Hill, Waterpoort, Northern Transvaal Province	20°54′ S 29°48′ E
E. edwardii	Nieuwoudtville, Northern Cape Province	31°23′ S 19°06′ E
E. intufi	Langjan Nature Reserve, Northern Transvaal Province	22°50′ S 29°15′ E
E. myurus	Deelfontein, Northern Cape Province	31°02′ S 23°46′ E
E. rupestris	Richmond, Northern Cape Province	31°10′ S 24°56′ E
M. proboscideus	Beaufort West, Western Cape Province	32°22′ S 22°33′ E
P. tetradactylus	False Bay, Kwazulu/Natal	27°57′ S 32°24′ E

Tissue homogenisation

Liver, kidney and heart tissue samples from individuals of each species were homogenised in an Optolabor ultra turrax (T25) at 0 °C. Liver and kidney samples were individually homogenised in phosphate buffered saline (pH 7,40) while heart samples were homogenised in 1,000 M KCl buffer. After homogenisation samples were centrifuged in a Beckman 200 E Microfuge (30 sec.; room temperature). Precipitates, containing cellular debris, were discarded while the supernatants containing active enzyme extracts were stored at 20 °C until needed.

Electrophoresis

Suitable dilutions of the enzyme extracts from each species were subjected to polyacrylamide gel electrophoresis using a Mighty Small SE 200 Electrophoresis unit. Polyacrylamide gels and electrode buffers were prepared according to the methods of either Hoefer (1993) or Krasfur (1993).

After electrophoresis, enzymatic activity was detected using various modifications of the stains suggested by Harris and Hopkinson (1978) and Selander et al. (1971). The enzymes tested for were: esterase 1 (ES 1; EC 3.1.1.1), esterase 2 (ES 2), esterase 3 (ES 3), fumarate hydrase 1 (FUM 1; EC 4.2.1.2), fumarate hydrase 2 (FUM 2), glucose dehydrogenase 1 (GD 1; EC 1.1.1.47), glucose-6-phosphate dehydrogenase 1 (G-6-PD 1; EC 1.1.1.49), glutamate oxaloacetate transaminase 1 (GOT 1; EC 2.6.1.10), glycerophosphate dehydrogenase 1 (GPD 1; EC 1.1.1.8), isocitrate dehydrogenase 1 (ICD 1; EC 1.1.1.42), isocitrate dehydrogenase 2 (ICD 2), lactate dehydrogenase 1 (LDH 1; EC 1.1.1.27), lactate dehydrogenase 2 (LDH 2), lactate dehydrogenase 3 (LDH 3), lactate dehydrogenase 4 (LDH 4), malate dehydrogenase 1 (MDH 1; EC 1.1.1.37), malate dehydrogenase 2 (MDH 2), malic enzyme 1 (ME 1; EC 1.1.1.40), mannose phosphate isomerase 1 (MPI 1; EC 5.3.1.8), phosphogluconate dehydrogenase 1 (PGD 1; EC 1.1.1.44), phosphogluconate dehydrogenase 2 (PGD 2), phosphoglucomutase 1 (PGM 1; EC 5.4.2.2), phosphoglucose isomerase 1 (PGI 1; EC 5.3.1.9), superoxide dismutase 1 (SOD 1; EC 1.15.1.1) and xathine dehydrogenase 1 (XDH 1). Staining of the polyacrylamide gels was achieved by using a solid substrate medium rather than the more commonly used liquid medium, as the former was found to be both more sensitive and more cost effective. The solid substrate medium was an agar gel (2%) containing substrate(s), essential cofactors and dye ingredients. The polyacrylamide gels were placed on top of a substrate gel and all the gels were incubated at 37 °C in the dark until bands appeared on the polyacrylamide gels (with the exception of esterases). For esterase staining Fast Blue Dye and naphthyl was added to agar. The polyacrylamide gels were then placed on top of the substrate gels, incubated at room temperature until satisfactory staining was attained. The stained polyacrylamide gel was then washed in fixative, fixed overnight and then the gels were dried on to filter paper and analysed. The stained polyacrylamide gels were then placed on to filter paper, air dried (24 h) and analysed.

Analysis of Data

The bands obtained on the polyacrylamide gels were initially termed electromorphs until a genetic basis for them was confirmed. Confirmation was achieved using the following procedure:

1) the number of bands detected in a heterozygous individual for each polymorphic locus was compared to known patterns of the enzyme in a polymorphic species e. g. man.
2) checks were made for known numbers of subunits for that enzyme in other species.

If these two criteria were met, then the electromorph was referred to as an allele. Alleles were coded according to their mobility from the origin with the most anodal allele coded "a" and successively more cathodal alleles as "b", "c" and so on until all the alleles were named. Adjacent comparisons of the alleles obtained from the enzyme extracts of the liver, heart and kidney tissues from individuals of the elephant shrew species were performed on all stains to ensure correct allelic designation. Statistical calculations were done using the BIOSYS 1 computer program of Swofford and Selander (1989).

Results

Results from tests for isozymes and allozymes from the three different tissue types (heart, liver, and kidney) from seven elephant-shrews species proved interesting. Although most of the loci tested displayed the same number of isozyme(s) and allozyme(s) in all three tissue extracts in all species e. g. MDH 1, occasionally the number of isozymes obtained in the different tissues varied e. g. LDH. The extracts of heart tissues tested for LDH activity, showed the presence of only three anionic isozymes while both the kidney and liver extracts showed the presence of all four anionic isozymes.

Statistical analysis of the allozymes and isozymes data was restricted to the data obtained from liver extracts, since this tissue produced the clearest and most reproducible

staining reactions for all loci tested. The allozymes and isozymes obtained from 26 presumptive loci are listed in table 2. Unfortunately the isozymes and allozymes for XDH could not be resolved in any of the tissue extracts tested, despite many various attempts at staining using different buffers and gel systems.

Table 2. Allelic designations of 23 genetic loci for seven elephant-shrew species.

LOCUS	SPECIES						
	*Eb**	*Ee**	*Ei**	*Em**	*Er**	*Mp**	*Pt**
ES 1	A	A	A	A	A	A	A
ES 2	A	A	A	A	A	A	A
ES 3	A	A	A	A	A	A	A
FUM 1	C	A	B	D	C	E	E
FUM 2	C	A	D	E	B	B	C
GD 1	**A**, D	C	B	C	C, **A**	E	E
G-6-PD 1	C	D	**B**, C	**B**, C	C	**A**, D	D
GOT 1	C	B	B	B	A	D	E
GPD 1	C	B	C	B	C	B	C
ICD 1	B	D	A	C	C	B	D
ICD 2	E	D	A	D	B	B, C	C
LDH 1	A	A	A	A	A	A	A
LDH 2	A	A	A	A	A	A	A
LDH 3	A	A	A	A	A	A	A
LDH 4	A	A	A	A	A	A	A
MDH 1	A	A	A	A	A	A	A
MDH 2	A	A	A	A	A	A	A
ME 1	C	C	A	C	B	C	C
MPI 1	B	A	D	C	E	C	C
PGD 1	A, **D**	A	B	C	D	D	D
PGD 2	D	C	A	B	B	E	E
PGM 1	B	A	C	B	C	C	A
PGI 1	B	A	B	B	A	C	C
SOD 1	B	A	B	B	A	C	C
SDH 1	D	C	A	B	A	F	E
XDH 1	–	–	–	–	–	–	–

**Eb – E. brachyrhynchus, Ee – E. edwardii, Ei – E. intufi, Em – E. myurus, Er – E. rupestris, Mp – M. proboscideus, Pt – P. tetradactylus.*
Bold alleles are the rarer forms found in the test sample.

Of the 26 loci examined, nine loci, LDH 1–4, MDH 1–2 and ES 1–3, were fixed in all the elephant-shrew species tested. Glucose dehydrogenase was found to be polymorphic in *E. brachyrhynchus* and *E. rupestris* while G-6-PD 1 was polymorphic in *E. brachyrhynchus*, *E. intufi*, *E. myurus*, and *M. proboscideus*. Only one individual from all the *E. brachyrhynchus* tested, possessed a different allele at the locus PGD 1 (Tab. 2) and one individual from all the *M. proboscideus* tested possessed a different allele at the locus ICD 2.

The degree of heterozygosity (H) was relatively low (Tab. 3), with the mean H varying from 0.000 in *P. tetradactylus* and *E. edwardii* to 0.055 in *E. brachyrhynchus* (Tab. 3). The H values of the other species tested were found in the midrange with *E. edwardii* possessing the lowest H (0.021) of this group. Like the degree of heterozygosity, the degree of polymorphism was relatively low, with *E. brachyrhynchus* showing the highest percentage polymorphism (12.50%) (Tab. 3).

Table 3. Mean heterozygosity (H) and percentage polymorphism (% P) in seven elephant-shrew species.

SPECIES	NUMBER OF INDIVIDUALS	% P	H ± SE
E. brachyrhynchus	10	12.50	0.055 ± 0.000
E. edwardii	10	0.00	0.000 ± 0.000
E. intufi	3	6.30	0.033 ± 0.033
E. myurus	10	6.30	0.021 ± 0.021
E. rupestris	10	6.30	0.032 ± 0.032
M. proboscideus	10	6.30	0.032 ± 0.032
P. tetradactylus	4	0.00	0.000 ± 0.000

The degree of similarity and difference between the species, using both Nei's (1978) genetic identity (I) and genetic distance (D) are shown in table 4. The values of D varied from 0.323 (between *M. proboscideus* and *P. tetradactylus*) to 0.970 (between *E. myurus* and *P. tetradactylus*). The values obtained using Nei's identity calculations produced a similar pattern to that of the distance values (Tab. 4). The D values between the *Elephantulus* species are quite large implying a high degree of divergence. The D value between *M. proboscideus* and *P. tetradactylus* is relatively low (0.323), implying a close relationship between these two species. The values obtained for Roger's (1972) genetic distance and genetic similarity calculations are shown in table 5. Although they mirrored the general

Table 4. Values of Nei's genetic identity (I, above the diagonal) and genetic distance (D, below the diagonal) between seven elephant-shrew species

SPECIES	*Eb**	*Ee**	*Ei**	*Em**	*Er**	*Mp**	*Pt**
*Eb**	–	0.469	0.473	0.464	0.511	0.465	0.469
*Ee**	0.756	–	0.445	0.505	0.483	0.406	0.500
*Ei**	0.749	0.810	–	0.553	0.474	0.388	0.381
*Em**	0.768	0.683	0.593	–	0.565	0.385	0.379
*Er**	0.671	0.728	0.747	0.571	–	0.516	0.445
*Mp**	0.765	0.9000	0.948	0.954	0.661	–	0.724
*Pt**	0.756	0.693	0.964	0.970	0.811	0.323	–

**Eb – E. brachyrhynchus, Ee – E. edwardii, Ei – E. intufi, Em – E. myurus, Er – E. rupestris, Mp – M. proboscideus, Pt – P. tetradactylus.*

Table 5. Values of Roger's genetic similarity (I, above the diagonal) and genetic distance (D, below the diagonal) between seven elephant-shrew species.

SPECIES	*Eb**	*Ee**	*Ei**	*Em**	*Er**	*Mp**	*Pt**
*Eb**	–	0.463	0.471	0.463	0.503	0.466	0.463
*Ee**	0.537	–	0.445	0.505	0.475	0.400	0.500
*Ei**	0.529	0.555	–	0.554	0.466	0.392	0.382
*Em**	0.537	0.495	0.446	–	0.550	0.389	0.380
*Er**	0.497	0.525	0.534	0.450	–	0.516	0.446
*Mp**	0.534	0.600	0.608	0.611	0.484	–	0.712
*Pt**	0.537	0.500	0.618	0.620	0.554	0.287	–

**Eb – E. brachyrhynchus, Ee – E. edwardii, Ei – E. intufi, Em – E. myurus, Er – E. rupestris, Mp – M. proboscideus, Pt – P. tetradactylus.*

pattern of the Nei values rather closely, they do not show as great a divergence amongst the species as predicated by the Nei's analysis.

Cluster analysis, employing the unpaired group method using the arithmetic mean analysis (UPGMA) of Nei's D and I values produced the phenograms shown in figures 1 and 2 respectively. Both phenograms divide the species into two main clusters which correspond to the *Elephantulus* and *Petrodromus/Macroscelides* groupings. The cluster containing *P. tetradactylus* and *M. proboscideus* appear to be far more closely related in terms of allozymic analysis, than initially thought. The *Elephantulus* grouping has *E. myurus* and *E. rupestris* the most closely related, with *E. edwardii* and *E. brachyrhynchus* the most divergent from this grouping (Figs. 1, 2).

The Wagner tree produced from the Roger's distance calculations, maintained the two broad genera clusters as shown by the Nei D and I calculations, but *E. brachyrhynchus* was classed within the *Macroscelides/Petrodromus* cluster (Fig. 3).

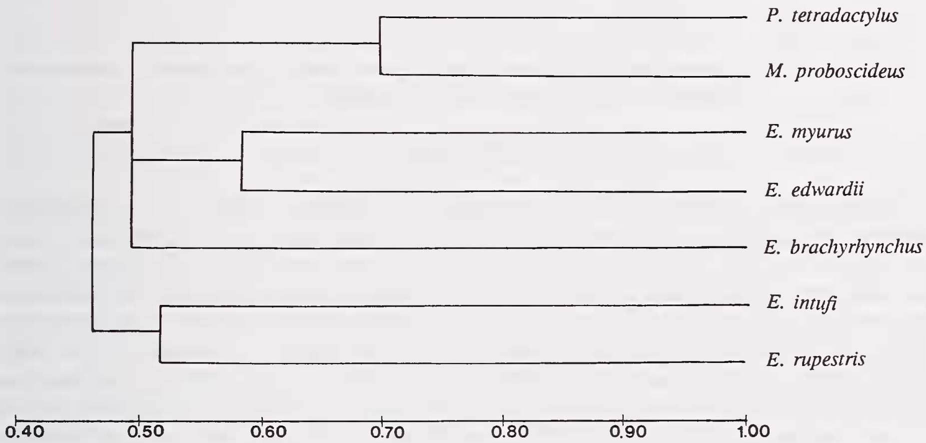

Fig. 1. Phenogram produced using UPGMA analysis of Nei's genetic identity of allozyme and isozyme data of seven elephant-shrew species (co-phenetic value = 0.864).

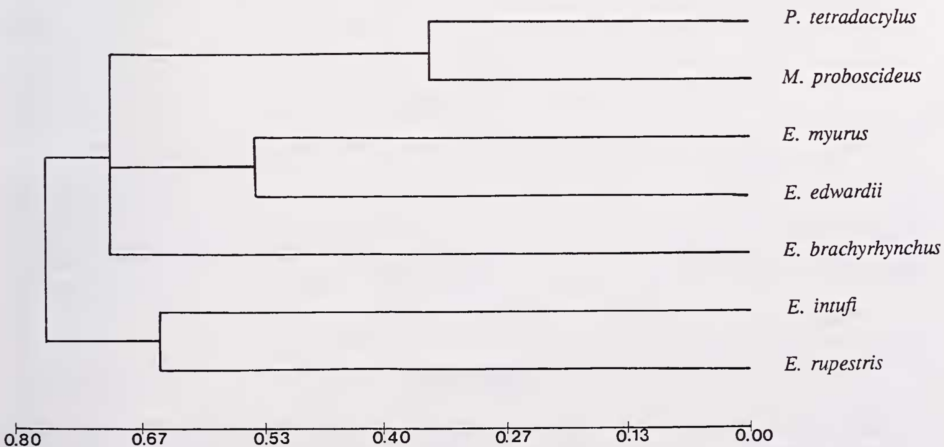

Fig. 2. Phenogram produced using UPGMA analysis of Nei's genetic distance for allozyme and isozyme data of seven elephant-shrew species (co-phenetic value = 0.820).

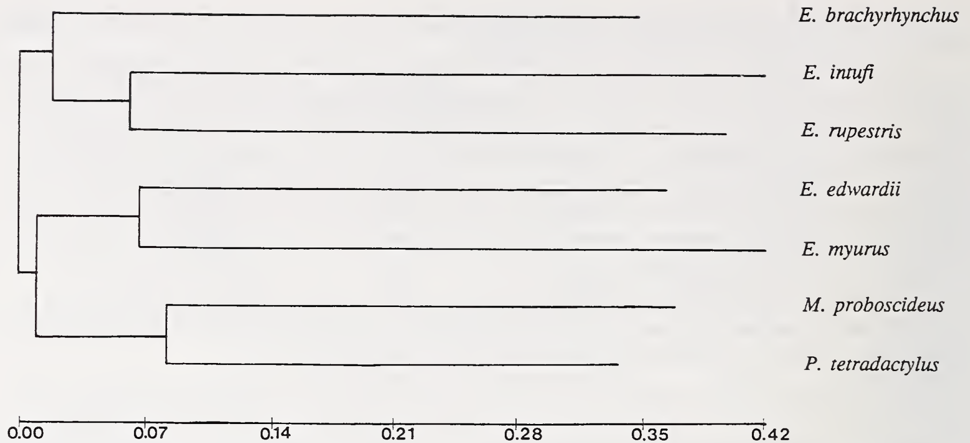

Fig. 3. Wagner tree produced using analysis of Roger's genetic distance of allozyme and isozyme data from seven elephant-shrew species (co-phenetic value = 0.818).

Discussion

When attempting allozyme analysis it is of vital importance to maintain and analyse different tissue extracts from each species, separately. Each tissue type has its own number and form of isozymes and allozymes and therefore when two different tissue types from the same species are mixed together, and subsequently analysed, the picture of isozyme and allozyme produced when subjected to electrophoresis is somewhat unclear, especially if they have different embryonic origins. Observed variations in the isozymes and allozymes patterns may be due to the differing concentrations of the tissues in the mixtures rather than due to real differences in the allozyme/isozyme patterns between different species. The mixing of tissues could be a possible reason for the discrepancy in the allelic results obtained by TOLLIVER et al. (1989) when compared to those of this study. TOLLIVER et al. (1989) used a mixture of two tissue types (heart and kidney) in their allozyme analysis of elephant-shrews and found more alleles per loci and a far greater degree of variation between the species.

In all the species sampled the percentage polymorphism and degree of heterozygosity was rather low. This could be due to the small sample size but it has been shown by GORMAN and RENZI (1979) as well as NEI (1978) that estimates of genetic distances obtained from data from a few animals do not vary significantly from those obtained from much larger sample sizes, providing a reasonable number of loci have been sampled.

The cluster/UPGMA tree produced from Nei's I values differed from the tree produced by TOLLIVER et al. (1989) as well as the dendrogram based on morphological features produced by CORBET and HANKS (1968). Unlike the trees produced in the previous studies, both *P. tetradactylus* and *M. proboscideus* appear to be closely related species rather than two distinct genera with I = 0.724 which is within the range for congeneric species suggested by AYALA (1975). Although the allozyme analysis suggests a close relationship, morphologically, behaviourally and chromosomally the two species are very distinct. Both of these species have very distinct habitats which do not overlap. These facts suggest that since these two species diverged from their common ancestor, selection pressure has been directed towards changes in morphology and behaviour patterns, making them better adapted to their specific environments rather than mutations occurring to prevent hybridisation between the two species. It is therefore suggested that these two species be maintained in two different genera.

The arrangement within the *Elephantulus* clustering using Nei's I values is interesting, with one of the most advanced species in this group being *E. myurus.*

Chromosomal analysis supports this, as the increase in this species's diploid chromosome number has been explained by the more advanced form of Robertsonian mutation, namely fission. Despite close morphological similarity between members of the genus, the Nei D and I values infer a great degree of genetic divergence between the species. A possible reason for this is that the morphological appearance adopted by the members of the *Elephantulus* cluster is the most evolutionary stable form for their general habitat and they have therefore diverged at the genetic level (chromosomes and allozymes) in order to prevent hybridisation.

Two basic clusters were also produced by the Wagner analysis of Roger's genetic distance but they differed from those produced by UPGMA analysis of Nei's genetic distance. In the Wagner analysis *E. edwardii* and *E. myurus* were classes with the *M. proboscideus* and *P. tretradactylus* grouping while *E. brachyrhynchus* was grouped with *E. intufi* and *E. rupestris.* This added credence to the argument that *E. brachyrhynchus* should be maintained in the genus *Elephantulus.* The accuracy of the genetic distance calculations and branch length estimates depends on the molecular clock hypothesis (Felsentein 1984) and many researchers are of the opinion that UPGMA analysis using Nei's genetic values best estimate the molecular clock. It is for this reason that the Wagner tree is disregarded. However, based on the UPGMA analysis of Nei's genetic distance and the fact that *E. brachyrhynchus* was maintained in the *Elephantulus* grouping by the Wagner analysis, it was decided to retain *E. brachyrhynchus* in the genus *Elephantulus* but as a species which had diverged from the common *Elephantulus* ancestor relatively early on in the development of this genus.

Allozyme analysis proved to be a good marker for the identification of different elephant-shrew species, since each species possessed its own unique allelic pattern. The allozyme analysis using Nei calculations also supported the general generic breakdown as well as the hypothesis that *E. brachyrhynchus* should remain within the *Elephantulus* genus rather than being placed in a separate genus. The phylogeny proposed by this study suggests that allozyme divergence within the elephant-shrew group has adopted two forms. The first occurs between the *Macroscelides* and *Petrodromus* genera, where the changes at the allozyme level have occurred more recently, most likely by genetic drift. The second occurs mainly in the *Elephantulus* taxon, where the allozyme divergence is primarily directed towards the prevention of hybridisation between the species.

Zusammenfassung

Allo- und Isoenzymvariation bei sieben Elefantenspitzmausarten des südlichen Afrika

Allo- und Isoenzymvariationsanalyse bei sieben Elefantenspitzmausarten aus dem südlichen Afrika zeigte in beiden Fällen artspezifische Muster. Die Auswertung, nach der Methode von Nei, ergab große Divergenz zwischen Angehörigen der Gattung *Elephantulus,* während sie zwischen den Arten *Macroscelides proboscideus* und *Petrodromus tetradactylus* gering war. Nach der UPMGA Analysemethode wurde *E. brachyrhynchus* als Mitglied der Gattung *Elephantulus* bestätigt.

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